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Full Length Research Paper

Staphylococcus aureus sequence type (ST) 1 isolated from sub clinical mastitis in settled Fulani herds in Kaduna State, Nigeria

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A study was carried out to monitor the sequence type of Staphylococcus aureus from bovine mastitic milk in Kaduna State, Nigeria. The dairy cows and fresh milk samples were analyzed for clinical and subclinical mastitis, followed by isolation of *S. aureus* and antimicrobial susceptibility testing. Twenty-eight *S. aureus* were isolated phenotypically from bovine subclinical mastitis which were further examined molecularly for the acquisition of *mec A, mec C* and *fem B* genes. All the isolates were negative for *mec A* and *mec C* genes, while 3 isolates were positive for *fem B* gene. Typing using MLST revealed that the 3 *fem B* positive isolates were Sequence Type 1 (ST 1) and clonal complex 1, which had similar allelic profiles at all seven loci and showed high degree of monomorphism. The 3 MLST ST 1 isolates were recovered from cases of subclinical mastitis. The antibiotic susceptibility profile showed that the 3 MLST ST 1 isolates were multidrug resistant and resistant to commonly used antimicrobials. The phylogenetic relationship established using the nucleotide sequences revealed that the sequence of the strains MT550650, MT550651 and MT550652 had 99 % identity with the *S. aureus* BX571857, BA000033, AP015012, CP017115 and CP01780 available in the database. The MT550650, MT550651 and MT550652 *S. aureus* strains were found to be 100% homologous to each other with no divergence within the specie level. The isolation of ST 1 *S. aureus* strains from milk is of public health significance due to their role in food poisoning. Therefore, the study recommends proper hygienic procedures to reduce the contamination of milk by *S. aureus* and possible prevention of its spread to other animals and human population.

Key words: *Staphylococcus aureus,* MLST, sequence type (ST), subclinical mastitis, settled Fulani herds, Kaduna State.

INTRODUCTION

Mastitis is a disease that causes loss in milk production due largely to decrease milk and milk products quantity and quality. Among aetiological agents of mastitis, *Staphylococcus aureus* is the most frequent causative agent in cattle (Islam et al., 2010; Junaidu et al., 2011; Intrakamhaeng et al., 2012; Shittu et al., 2012). *S. aureus* is an important species among the Staphylococci group that are dispersed (Mirzaei et al., 2011), and constitutes part of micobiota of the skin, intestine, upper respiratory tract and vagina of humans and animals (Chaibenjawong and Foster, 2011). *S. aureus* has the ability and capacity to acquire resistance against antibiotics and has now turned it into an organism of major concerns to public health (Helal et al., 2015). Methicillin resistance is through the *mec* operon, which is part of the staphylococcal cassette chromosome *mec* (SCC*mec*), and attributed to *mec* A gene, which codes for an altered penicillin-binding protein (PBP2a or PBP2') that has a lower affinity for binding β-lactams (Ba et al., 2014). However, a novel *mec* A homologue, known as *mec C* (previously *mecA*$_{LGA251}$) that also confers methicillin resistance was identified in *S. aureus* isolates from dairy cattle and humans (Pexara et al., 2013; Ba et al., 2014). The *fem* B gene causes the production of the pentaglycine interpeptide bridge, which is diagnostic of the *S. aureus* peptidoglycan and influence the level of methicillin resistance of *S. aureus* (Kobayashjf et al., 1994), while the housekeeping genes are constitutive genes needed to preserve the basal cellular functions to maintain the life of a cell, regardless of its specific role in the tissue or organism. Hence, they act as the minimal set of genes needed to sustain life (Enright et al., 2000).

S. aureus is the major and economically significant infectious agents responsible for intramammary infections in dairy herds (Pexara et al., 2013; Joshi et al., 2014), and has been the factor responsible for 30 to 40% mastitis cases (Botrel et al., 2010; Suleiman et al., 2012; Haran et al., 2012). Therefore, it contaminates, grows and multiplies in milk usually from udder resulting to clinical or subclinical mastitis or from environment during milking and processing (Botrel et al., 2010; Mirzaei et al., 2011; Suleiman et al., 2012; Li et al., 2017). Currently, multi-drug resistant *S. aureus* have become an increasing issue in dairy environment due to their widespread worldwide and possible transmission to humans (Shittu et al., 2014; Helal et al., 2015). Increasing drug resistance in *S. aureus* has been a great threat to both hospitals and community due to its related consequences. This worldwide dissemination and spread of resistance *S. aureus* can threaten the treatment of infected patients through lowering the chances of antibiotics selection for the treatment of the infections by *S. aureus* (Alian et al., 2012; Kreusukon et al., 2012).

In Nigeria, there are reports of constant abuse and misuse of antibiotics coupled with the absence of proper antibiotic surveillance system, hence need to evaluate the multi-drug resistant bacteria present in dairy environments so as to forestall their spread and transmission to humans.

The determination of the origin of causative agents and information on the appropriate treatment and control measures of bovine mastitis is of great importance from the public health and economic point of views (Li et al., 2017; Rodrigues et al., 2019). It is therefore, essential to comprehensively understand the molecular epidemiology of the pathogens for the detection of transmission methods and infection sources (Mongkolrattanothai, 2013; Li et al., 2017).

Molecular characterization need to be enhanced to be able to correctly identify microorganisms causing mastitis and also the genes which contributes to virulence in these organisms (Li et al., 2017). In fact, they will help in epidemiological studies and will provide basis for tracing of pathogens throughout the milk production chain. The characterization of the genetic diversity of *S. aureus* isolated from milk-producing cattle is highly important for a better understanding of the pathogen's dispersion pattern. These data may be of great worth in the development of more effective master plan to decrease infection cases.

Genotypical analysis of *S. aureus* from milk and bovine origin has been demonstrated across the globe using Pulsed Field Gel Electrophoresis (PFGE), *S. aureus* Protein A (*spa*) Typing, Multilocus Sequence Typing (MLST) and Staphylococcal Cassette Chromosome (SCC *mec*) typing (Kreusukon et al., 2012; Haran et al., 2012; Suleiman et al., 2012; Helal et al., 2015; Ali, 2016; Li et al., 2017; Rodrigues et al., 2019). In this study, multilocus sequence typing (MLST), a typing method based on the sequence polymorphism of fragments of seven housekeeping genes was used to determine the *S. aureus* sequence types and clonal lineages disseminated in the dairy environments in Kaduna State, Nigeria. At present, information is scarce on the genetic spread of *S. aureus* in bovine mastitis or dairy environments in Nigeria. Therefore, the research was aimed at determining the antibiogram, multidrug resistance nature

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and molecular characterization of *S. aureus* causing sub clinical mastitis in Kaduna State, Nigeria.

MATERIALS AND METHODS

Sample collection

Milk samples were aseptically collected within a six-month period (June to December 2017) from dairy cows with or no symptoms of clinical mastitis from settled Fulani herds in Sabon-Gari, Giwa and Zaria in Northern senatorial zone, Kaduna South and Igabi in the central senatorial zone and Kagarko in the Southern senatorial zone of Kaduna State, Nigeria. Manuel milking was performed after cleaning the teat ends with swabs that was moistened in 70% alcohol. The first streams (2 ml) of the milk samples were discarded; 5 ml of the composite milk samples were collected into sterile plastic sample bottle, placed on ice and transported to the Bacterial Zoonoses laboratory, Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria for the isolation of *S. aureus*.

Clinical and subclinical mastitis test

The observation of the udder visually of clinical mastitis was measured by palpation for noticeable trauma, tick infestation, pain, warmth and swelling of the super mammary gland (Mekibib et al., 2010). Abnormality in milk characteristics like blood tinged milk, watery secretions, clots and pus were checked (Dinwell et al., 2003). Cows that did not have clinical mastitis were subjected to further examination for subclinical mastitis by using California Mastitis Test (CMT) as described by Dinwell et al. (2003). Foremilk from each quarter was milked into cups of four-cup plastic paddle. The paddle was tilted to equalize milk quantities in the cups at 2.5 ml each. Equal volume of the CMT reagent (Kruuse, Denmark) was added to each cup. The paddle was rotated to mix thoroughly. Changes in colour and gel formation were observed within 10 to 15 s after mixing and then scored depending upon the amount of gel formation as follows:

No reaction= Negative,
Appearance of streaks visible during rotation of the plate= Trace,
Distinct thickening during rotation, but no gel= 1+,
Slight formation of gel which follows the rotating plate very slowly = 2+,
Solid formation of gel that adheres to the base of the plate= 3+.

Bacterial isolation

The milk samples (2 ml) collected was inoculated into 5 ml Tryptone Soya Broth (TSB, Oxoid, United Kingdom) containing 6.5% sodium chloride and was incubated for 24 h at 37°C for selective enrichment of staphylococci (Cheesbrough, 2010). The enriched milk samples were cultured in Baird Parker medium supplemented with egg yolk and potassium tellurite and incubated aerobically for 24 to 48 h at 37°C (Tamagnini et al., 2006; Cheesbrough, 2010). The *S. aureus* isolates were identified based on colony morphology, Gram staining, catalase, coagulase, haemolysis, DNase and fermentation of sucrose, glucose, lactose and mannitol, and were further identified using Microbact 12S Staphylococcal Identification System (Oxoid, Basingstoke, UK). *S. aureus* ATCC 33591 strains was used as positive control. A total of 28 *S. aureus* isolates were phenotypically recovered from sub clinical mastitis and were stored at -80°C in 10% glycerol TSB prior to further analysis. *S. aureus* isolates were inoculated onto Mannitol Salt agar and Tryptone Soya agar slants in cryo vials and also spotted on Whatman filter Paper, placed in a container with ice and shipped to The Wellcome Trust, Sanger Institute, United Kingdom for the molecular characterization.

Antimicrobial susceptibility testing

All the *S. aureus* isolates were tested for susceptibility to a panel of 14 antibiotics using the disc diffusion method outlined by the Clinical Laboratory Standard Institute (CLSI, 2014). Antibiogram classifications were made on the basis of susceptibility to 14 antimicrobials obtained from Oxoid (Oxoid Basingstoke, United Kingdom): amoxicillin (30 µg), ampicillin (30 µg), cefoxitin (30 µg), chloramphenicol (12 µg), ciprofloxacin (5 µg), erythromycin (5 µg), gentamicin (10 µg), nalidixic acid (30 µg), oxacillin (1 µg), penicillin (10 µg), streptomycin (10 µg), tetracycline (30 µg), trimethoprim (5 µg), and vancomycin (30 µg). Three colonies that were well isolated were selected from a nutrient agar plate culture, transferred to Brain Heart Infusion Broth (BHI) and then incubated at 35°C for 6 h for the turbidity to reach 0.5 McFarland standards (CLSI, 2014). A sterile cotton swab stick was dipped into inoculum suspension and swabbed several times on the dried surface of the Mueller-Hinton Agar (Ryan and Ray, 2004). The antibiotic discs were then applied using the disk dispenser (Oxoid Basingstoke, United Kingdom). The plated discs were allowed to dry and incubated at 35°C for 24 h. Zones of inhibition were observed in some plates and the diameters measured to the nearest whole millimeter using a ruler. The sizes of the zones of inhibition were interpreted by comparing with the normal breakpoints (CLSI, 2014). *S. aureus* ATCC 33591 strains was used as positive control.

mec A, *mec* C and *fem* B genes detection by multiplex polymerase chain reaction

DNA extraction

One or two colonies of bacterial cultures were picked from overnight growth cultures on blood agar and suspended with 40 µl water (DNase free water). This was then heated for 5 min at 95°C in a thermocycler (McLauchlin et al., 2000; Ali, 2016). Cured lysate mixture (2.5 µl) was used as a DNA template for Multiplex PCR procedures.

PCR procedures

Multiplex Polymerase Chain Reaction to amplify *mec* A, *mec* C and *fem* B genes were performed using DNA template, with the following amplification mixture: 200 µM of dNTPs, 1.5 mM magnesium chloride (Promega Corporations, USA), 0.25 µM of both forward and reverse primers of each gene, and 5 µL of template in a total volume of 50 µL. The amplification was carried out in MyCycler Thermal Cycler (Bio-Rad) with an initial denaturation at 94°C for 30 s, 30 cycles of denaturation temperature of 94°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min, and final extension at 72°C for 2 min. *S. aureus* ATCC43300 and ATCC25932 were used as positive and negative controls, respectively.

Agarose gel electrophoresis

Molecular biology grade Agarose (2% w/v) was dissolved by

Table 1. Oligonucleotide primers of *S. aureus mec* A, *mec* C and *fem B* genes.

Name	Sequence (5'-3')	Size (bp)	Gene	Reference
mecCA-F *mecCA-R*	CAT TAA AAT CAG AGC GAG GC TGG CTG AAC CCA TTT TTG AT	188	*mec*C	Paterson et al. (2014)
mecA-F *mecA-R*	TGG TAT GTG GAA GTT AGA TTG GGA T CTA ATC TCA TAT GTG TTC CTG TAT TGG C	155	*mec*A	Nakagawa et al. (2005)
femB-F *femB-R*	CAT GGT TAC GAG CAT CAT GG AAC GCC AGA AGC AAG GTT TA	531	*fem*B	Paterson et al. (2014)

Table 2. Sequences of primers used in the PCR.

Gene	Primer	Sequence (5′-3′)
Carbamate kinase (*arcC*)	*arcC*-Up *arcC*-Dn	TTGATTCACCAGCGCGTATTGTC AGGTATCTGCTTCAATCAGCG
Shikimate dehydrogenase (*aroE*)	*aroE*-Up *aroE*-Dn	ATCGGAAATCCTATTTCACATTC GGTGTTGTATTAATAACGATATC
Glycerol kinase (*glpF*)	*glpF*-Up *glpF*-Dn	CTAGGAACTGCAATCTTAATCC TGGTAAAATCGCATGTCCAATTC
Guanylate kinase (*gmk*)	*gmk*-Up *gmk*-Dn	ATCGTTTTATCGGGACCATC TCATTAACTACAACGTAATCGTA
Phosphate acetyltransferase (*pta*)	*pta*-Up *pta*-Dn	GTTAAAATCGTATTACCTGAAGG GACCCTTTTGTTGAAAAGCTTAA
Triosephosphate isomerase (*tpi*)	*tpi*-Up *tpi*-Dn	TCGTTCATTCTGAACGTCGTGAA TTTGCACCTTCTAACAATTGTAC
Acetyl coenzyme A acetyltransferase (*yqiL*)	*yqiL*-Up *yqiL*-Dn	CAGCATACAGGACACCTATTGGC CGTTGAGGAATCGATACTGGAAC

heating in a microwave in 1×TAE buffer (40 mM Tris, 20 mM Acetate and 2 mM EDTA pH 8.1). Sybr® Safe DNA gel stain (Thermo Fisher, UK) was for agarose gel staining. Each 100 ml of 2% agarose contained 2.5 µL of Sybr® Safe which was poured into the gel tray (with 1 mm 25 well comb from PeqLab) to set. A 10 µL of PCR reaction mixture was loaded on to each well. The molecular marker (5 µL) used was HyperLadder™ 100 bp, consisting of 100 to 1000 bp ladder from Bioline, United Kingdom. Electrophoresis was performed in a Sigma-Aldrich electrophoresis tank with 1 ×TAE at 80 V for 75 minutes. Electrophoresed gels were visualized under blue-light and their images captured using the GelDoc™ XR System Imager (BioRad).

Multilocus sequence typing (MLST)

The sequences of the gene products of the primers and the DNA sequences of 7 housekeeping genes were compared with those in the EMBL/GenBank database by using BlastP (*http://www.ncbi.nlm.nih.gov/blast/blast.cgi*). Each primer pair amplified an internal fragment of the housekeeping gene (about 500 bp) and was allowed accurate sequencing of ~450-bp fragments of each gene on both strands (Enright et al., 2000).

The seven housekeeping genes shown in Table 2 were used in the final MLST scheme and the fragments were amplified by using the primers shown in Table 1.

PCRs were carried out with 50 µl reaction volumes containing 0.5 µl of chromosomal DNA, 0.5 µg of each primer, 1 U of *Taq* DNA polymerase (Qiagen, Crawley, United Kingdom), 5 µl of 10× buffer (supplied with the *Taq* polymerase), and 0.2 mM deoxynucleoside triphosphates (Perkin-Elmer Applied Biosystems; Foster City, California). The PCR was performed in a PTC-200 DNA engine (MJ Research, Boston, Mass) with an initial 1 min denaturation at 95°C, followed by denaturation at 95°C for 5 min, 30 cycles of annealing at 55°C for 1 min, extension at 72°C for 1 min, followed by a final extension step of 72°C for 5 min. The amplified products were

precipitated with 20% polyethylene glycol-2.5 M NaCl, re-suspended in cold 70% ethanol, and re-precipitated; and the sequences of both strands were determined with an ABI Prism 377 DNA sequencer with Big Dye fluorescent terminators and the primers used in the initial PCR amplification (Enright et al., 2000).

For each locus, the sequences obtained from all the isolates were compared and the different sequences were assigned allele numbers. For each isolate, the alleles at each of the seven loci defined the allelic profile which corresponded to its ST. The clustering of isolates was achieved by the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) from the matrix of the percentage of pair wise differences between the allelic profiles of the isolates by using Statistica (StatSoft, Tulsa, Oklahoma). The non-randomness in the distribution of variable sites along the sequence of each gene fragment was examined by the method of Sawyer (Sawyer, 1989). Polymorphic sites were displayed by using Sequence Output, a Macintosh program available from the MLST website (*http://mlst.zoo.ox.ac.uk)*.

The origins of the strains were examined by the algorithm eBURST, in order to group the received allelic profiles into clonal complexes. Grouping was based on the similarity between sequence types in six of seven loci and singleton STs are defined as not sharing six out of seven loci with any other STs in the data set. Comparison with all *S. aureus* isolates in the database at http://www.*mlst.net* revealed the presence of only one clonal complex (CC1).

Phylogenetic studies and analysis of the isolates

The phylogenetic relationship was established using the nucleotide sequences of the type strains defining the genus *Staphylococcus* BX571857, BA000033, AP015012, CP017115 and CP01780. The Blast search for previously reported sequences that are identical to the three (3) local isolates was done using NCBI GenBank (*http://www.ncbi.nlm.hih.gov/*). Neighbor-joining trees (Saitou and Nei, 1987) were constructed on the basis of genetic distances, estimated by Kimura's (1980) two-parameter method, using MEGA 5 (Kumar et al., 2001; *http://www.megasoftware.net*). In addition, the nucleotide sequences of three (3) *S. aureus* isolates (23448_1#128, 23448_1#129, 23448_1#131) were extracted, and a neighbor joining phylogenetic tree was constructed with Mega v.6.06 (Figure 1). The methods produced a topology revealing species clustering of *S. aureus* obtained in this study and those obtained from the Genbank. This further consolidated the molecular identities of the *S. aureus* that were found in this study. *Enterococcus faecium* strain SM KX430929 was used as an out group in the tree. The reliability of the trees was estimated by bootstrap confidence values (Felsenstein, 1985) and 1000 bootstrap replications were used. A bootstrap value of 70% was considered significant evidence for phylogenetic grouping.

Data analysis

The results of MLST analysis of the three strains were compared to a database of *S. aureus* STs available on-line (*http://saureus.mlst.net*). The sequences were processed using CLC Main Workbench 7 (CLC Bio-Qiagen, Denmark) and analyzed using the *S. aureus* MLST database to assign the allele type and thus the ST. The profiles obtained including the new alleles and new profiles were submitted to the MLST database to contribute to the resource for *S. aureus* global epidemiology.

Sequence comparisons were carried out using BIOEDIT (Version 7.0.9.0) (Ali, 2016). The sequences were processed using CLC Main Workbench 7 (CLC Bio-Qiagen, Denmark) and analyzed using the *S. aureus* MLST database to assign the allele type and thus the ST. The profiles obtained including the new alleles and new profiles were submitted to the MLST database to contribute to the resource for *S. aureus* global epidemiology.

Neighbor-joining trees (Saitou and Nei, 1987) were constructed on the basis of genetic distances, estimated by Kimura's (1980) two-parameter method, using MEGA 5 (Kumar et al., 2001; http://www.megasoftware.net). The reliability of the trees was estimated by bootstrap confidence values and 500 bootstrap replications were used.

RESULTS

mec A, *mec* C and *fem* B genes detection, MLST and antibiotic susceptibility tests

Of the 28 phenotypically isolated *S. aureus* examined for the acquisition of *mec* A, *mec* C and *fem* B genes, none is positive for *mec* A and *mec* C genes. Only three (3) of the isolates (23448_1#128, 23448_1#129 and 23448_1#131) were positive for *fem* B gene (Figures 1 and 2).

MLST typing revealed that the 3 *fem* B positive *S. aureus* isolates were MLST type 1 (ST 1) and clonal complex 1, which had similar allelic profiles at all seven loci (Tables 3 and 4). Hence, they showed high degree of monomorphism. All the three (3) isolates were isolated from cases of subclinical mastitis (Table 5).

The antibiotic susceptibility testing showed the 3 isolates were resistant to amoxicillin, ampicillin, cefoxitin, erythromycin, nalidixic acid, oxacillin, penicillin and tetracycline (Table 5). The 3 *fem* B positive *S. aureus* strains were found to be resistant to more than 3 antimicrobials and thus are regarded as multidrug resistant strains (Table 5).

Phylogenetic studies of the isolates

The phylogenetic relationship was established using the nucleotide sequences of the type strains defining the genus *Staphylococcus* BX571857, BA000033, AP015012, CP017115 and CP01780. A blastn search of all nucleotide sequence of MT550650, MT550651 and MT550652 (Umaru et al., 2020) using default parameters revealed 99% identity with the available *S. aureus* BX571857, BA000033, AP015012, CP017115 and CP01780 genomes in the database (Figure 3). The tree illustrates the close relationship of *S. aureus* MT550650, MT550651 and MT550652 with *S. aureus* isolates BX571857, BA000033, AP015012, CP017115 and CP01780 (Figure 3). It can also be shown from the tree that all the MT550650, MT550651 and MT550652 *S. aureus* obtained were 100% homologous to each other with no divergence within the species level (Umaru et al., 2020). They clustered together more tightly as compared to those obtained from Genbank. The sequence of

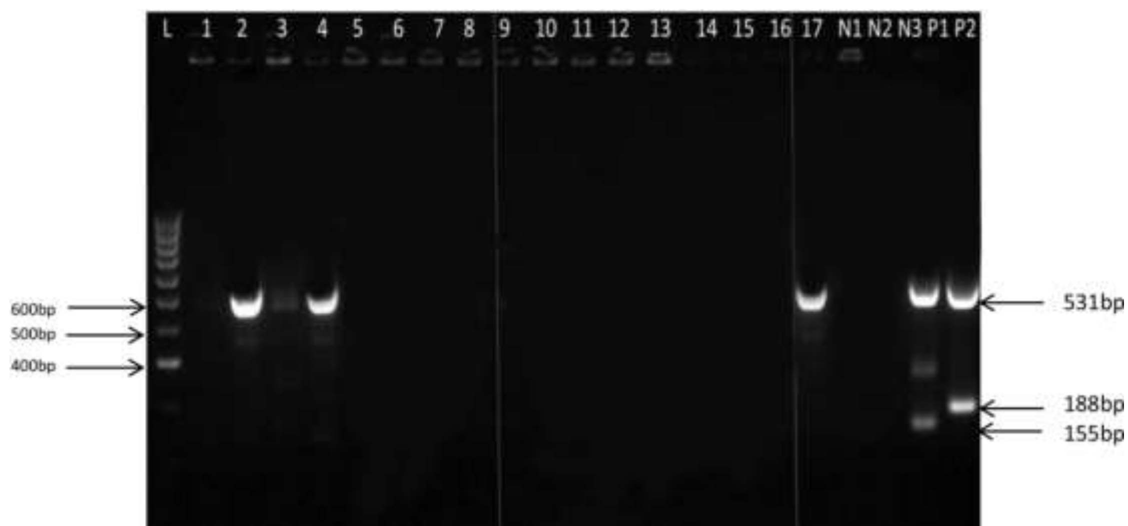

Figure 1. *mec* A (188 bp), *mec* C (155 bp) and *fem* B 531 (bp). Multiplex PCR. L= Molecular Ladder, N1= *mec* A negative control, N2= *mec* C negative control, N3= *fem* B negative control, P1= *fem* B positive control, P2= *mec* A positive control and number 1 to 17 are samples both positive and negative. Lane 2, 4 and 17 are *fem* B positive (23448_1#128, 23448_1#129 and 23448_1#131).

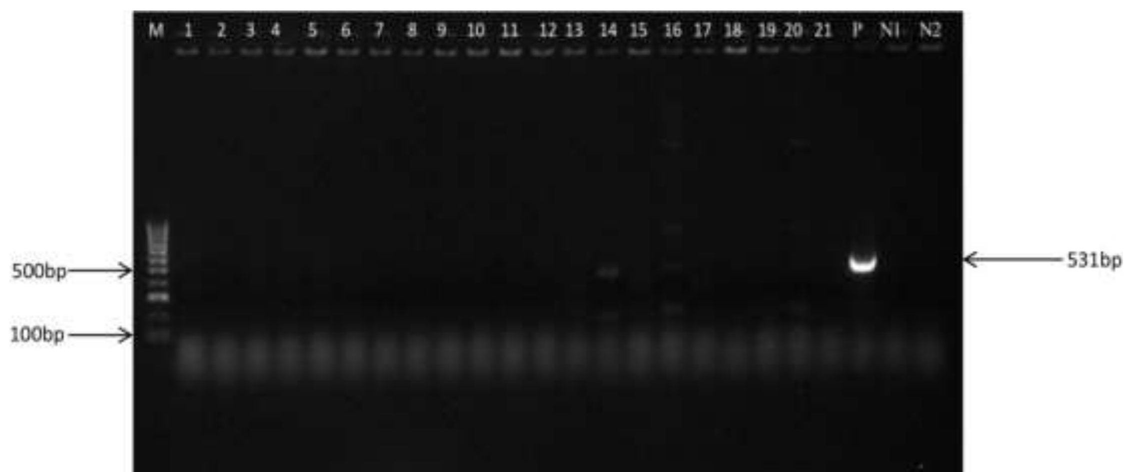

Figure 2. M= Molecular ladder, Lane 1-21= Samples P= *fem* B positive control N1 and N2= *fem* B negative controls. All isolates are negative for *mec* A, *mec* C and *fem* B genes.

Table 3. Sequence variation at the seven loci.

Gene	Sequence length (bp)	No. of alleles	No. of polymorphic sites
Arc	456	17	No
aroE	456	17	No
glpF	465	11	No
Gmk	429	11	No
Pta	474	15	No
Tpi	402	14	No
yqiL	516	16	No

Table 4. Properties of the 23448_1#128, 23448_1#129 and 23448_1#131sequence types.

Isolate	ST	Allelic profile (allele no) [a]							CC	Origin
		*aro*C	*aro*E	*glp*F	*Gmk*	*Pta*	*Tpi*	*yqi*L		
23448_1#128	1	1	1	1	1	1	1	1	1	Community
23448_1#129	1	1	1	1	1	1	1	1	1	Community
23448_1#131	1	1	1	1	1	1	1	1	1	Community

[a]Allelic profiles are read from left to right and correspond to genes *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *ygiL*. [b]CC, clonal complex.

Table 5. Resistance pattern and Multi-locus sequence type (MLST) of the isolates.

Isolate	Type of mastitis	Resistance profile	MLST
23448_1#128	Subclinical	AML,AMP,CN,E,FOX,NA, OX,P,S,TE	1
23448_1#129	Subclinical	AML,AMP,E,FOX,NA,OX,P,TE,VA,W	1
23448_1#131	Subclinical	AML,AMP,CN,E,FOX,NA,OX,P,TE,VA,W	1

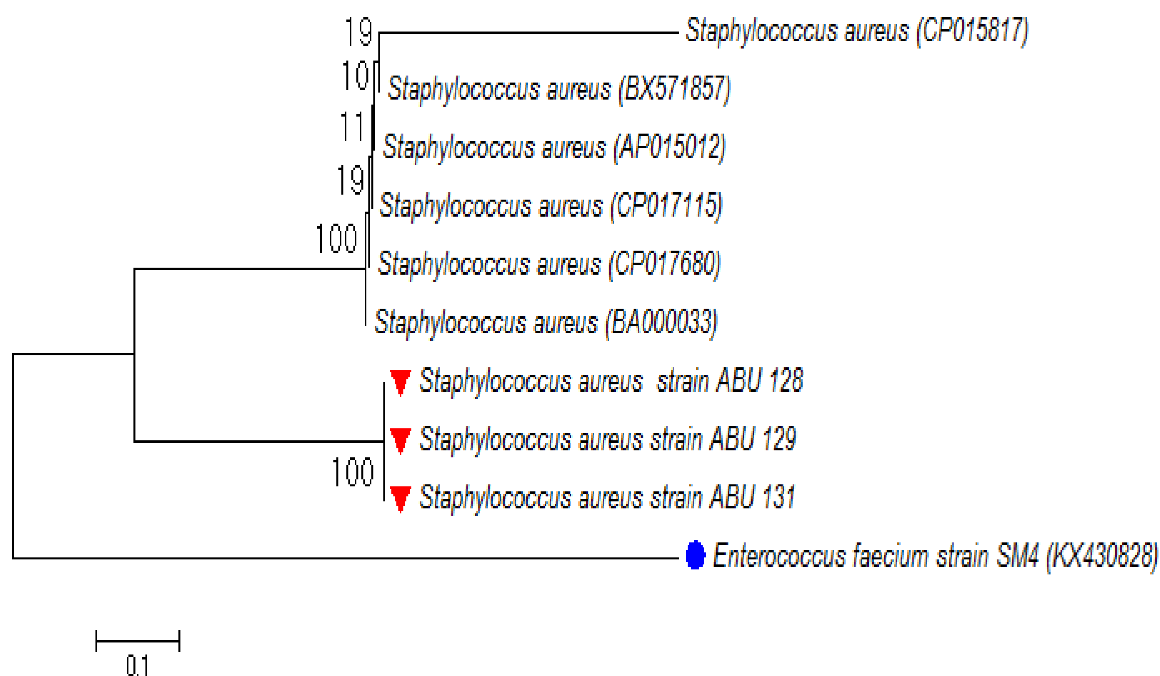

Figure 3. Neighbour-joining phylogenetic tree (1000 bootstrap replicates) of *Staphylococcus aureus* based on nucleotide residues of the ABU 128 (MT550650), ABU 128 (MT550651) and ABU 131 (MT550652) genes computed using the p-distance model (Felsenstein, 1985; Saitou and Nei, 1987). The *Staphylococcus aureus* haplo types isolated from milk in this study are indicated (ABU 128, ABU 128 and ABU 131 series) and those *S. aureus* obtained from the NCBI GenBank are with the accession numbers in parenthesis. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

S. aureus from the present study were in different branch from those in the GenBank signifying a significant level of within specie divergence between our isolates with those of the Genbank.

DISCUSSION

Milk is a good medium for the growth and multiplication of several bacteria among which staphylococci ranks the

first (Alian et al., 2012; Aliyu et al., 2019). Contamination of milk by staphylococci is normally through infection of the mammary gland via teat canal, milkers, milking equipment and from the environment (Alian et al., 2012). The contamination and presence of staphylococci are of public health significance due largely to their role in food poisoning. *S. aureus* is the most common pathogens associated with bovine mastitis, which is considered a disease of public health and economic importance, worldwide (Islam et al., 2010; Intrakamhaeng et al., 2012; Shittu et al., 2012).

In this study, 28 *S. aureus* isolates were phenotypically recovered from subclinical bovine mastitic milk collected from settled Fulani herds in Kaduna State, Nigeria and examined. Only 3 of the phenotypically recovered isolates (23448_1#128, 23448_1#129 and 23448_1#131) were *fem* B positive, while none was positive for *mec* A and *mec* C, respectively (Figure 1). The result of the present study is not different from others with respect to the occurrence of *fem* B among all isolates. For example, Ali (2016) found 13 of the 35 samples to be positive for *fem* B gene, while Kobayashjf et al. (1994) in their report showed that 18 strains (17.8 %) were *fem* negative methicillin-sensitive *S. aureus* and one (1I.1%) *fem* B positive *Staphylococcus epidermidis* were also detected.

However, it has also been recognized that detection of certain markers which are specific for *S. aureus* is needed to distinguish MRSA from methicillin-resistant CNS, in addition to demonstrating the *mec* A gene by PCR. Besides the *mec* regulator genes, *fem* A and *fem* B genes on the chromosome have been shown to encode proteins which considerably affect the level of methicillin resistance of *S. aureus* (Kobayashjf et al., 1994). Although, *fem* genes were suggested to be specific for *S. aureus* (Kobayashjf et al., 1994), distribution of these genes in staphylococci has not been fully established.

The 3 *fem* B positive *S. aureus* isolates were found to be resistant to more than 5 antimicrobials, and therefore had significantly higher multiple antibiotic resistance profiles. The finding is of serious concern considering the fact that the isolates were resistant to penicillin, ampicillin and tetracycline which are first-line and commonly available drugs in Nigeria. This is in consonant with the report of Aliyu et al. (2019) who also reported the emergence of multidrug resistant *S. aureus* in Nasarawa State, Nigeria in fresh and fermented milk. The detection of resistant *S. aureus* strains especially the beta lactam antibiotics is of public health concern. This is of serious concern as the treatment and control of subclinical mastitis in this area may likely fail due to the complex characteristics of the *S. aureus* strains. The extensive resistance of cattle-derived *S. aureus* will be a serious threat to bovine mastitis therapy. It has been reported that determinants of multi-drug resistance can be spread in a region or between regions due to antibiotic selective pressure in either humans or animals. The public health implication is that, antibiotic resistant strains of *S. aureus* from contaminated mastitic milk can be spread to humans through consumption or contact as occupational hazard.

MLST typing from this study revealed that all the 3 *fem* B positive isolates were ST 1 and clonal complex (CC 1), which had similar allelic profiles at all seven loci (1 1 1 1 1 1 1) (Tables 1 and 2). They were all recovered from cases of subclinical mastitis (Table 2). *S. aureus* is one of the main challenges in dairy farm industry and considered one of the main agents causing subclinical mastitis (Ali, 2016). This study agrees with several others in which ST1 strain was detected. For example, Rhee and Woo (2010) also detected ST1 in foods in strain Korea and reported that the gene which is an ST1 strain was the most epidemic clone found in staphylococcal food poisoning incidents in Korea, and may suggest the evidence of considerable transmission of antimicrobial-resistant pathogens and the origin of staphylococcal food poisoning in Korea. Tegegne et al. (2017) also detected ST1 (CC1) in cattle in the Czech Republic, which are commonly identified in Europe and are considered as human strains. Detecting human strains from food of animal origin may be a sign of cross-contamination during processing (Weese et al., 2010). However, colonization of livestock with human strains and jumping of animal strains to human host are increasing. In addition to this, host specific strains are widening their host range. Of recent, Dittmann et al. (2017) typed 66 *S. aureus* isolates by MLST to their persistent or continuously reintroduction in Brazilian Dairy Industries. Seven known sequence types (STs), ST1, ST5, ST30, ST97, ST126, ST188 and ST398, and four new ST were identified, ST3531, ST3540, ST3562 and ST3534. Clonal complex (CC) 1, known as an epidemic clone, was the dominant CC. However, there were no indications of persistence of particular ST (Dittmann et al., 2017).

According to the MLST database and previous studies on milk and dairy products, ST1 was not previously isolated in Nigeria, but was reported in other countries (Rhee and Woo, 2010; Silva et al., 2013; Dittmann et al., 2017). Numerous studies have discovered large variety concerning *S. aureus* contamination of milk. For example Silva et al. (2013) found that CC1 (ST1) was the main contaminant of milk in cows infected with mastitis due to MSSA and also identified a new ST belonging to CC1 in most countries of the world. This present information is the first report to the best of the author's knowledge and represents a preliminary study on ST 1 associated with bovine mastitis in Nigeria. The only available studies are the ones reported by Ghebremedhin et al. (2008) in Southwest Nigeria and Shittu et al. (2014) in Ile-Ife, Ibadan and Lagos (South-West Nigeria), and Maiduguri (North-East Nigeria). Both studies worked on human clinical samples from hospitals, but detected ST1 clones which were identified as community-acquired strains.

The phylogenetic relationships were established using

the nucleotide sequences of the type strains defining the genus *Staphylococcus* (BX571857, BA000033, AP015012, CP017115 and CP01780). A blastn search of all nucleotide sequence of MT550650, MT550651 and MT550652 using default parameters revealed 99% identity with the available *S. aureus* (BX571857, BA000033, AP015012, CP017115 and CP01780) genomes in the database (Figure 3). It can be inferred that the MT550650, MT550651 and MT550652 isolates and those obtained from database might have originated from common ancestry. From the tree, it can be seen that all the isolates clustered more tightly together as compared to those obtained in Genbank. They were 100% homologous to each other with no divergence within the species level indicating that they originated from the same ancestry or phylogeny. The present study is similar to that of Ali (2016) who performed the phylogenetic analysis of *S. aureus* and showed high degree of similarities between the local isolates of *S. aureus* those retrieved from the Gene Bank. Similarly, Zubair et al. (2015) determined the sequence type of the *S. aureus* isolate ILRI_Eymole1/1 from a Kenyan dromedary camel and a BLASTn search of all five copies of 16S rRNA sequence using default parameters revealed 99 to 100% identity (with 98-10% coverage) with all available *S. aureus* genomes in the database.

Therefore, improving the general herd hygiene, milking environment hygiene, personal hygiene and proper washing of containers and utensils can reduce the contamination of milk by *S. aureus* and possibly prevent its spread to other animals and human population.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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